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13. ABSTRACT *(Maximum 200 Words)*

The overall goal of my grant proposal is to test the model, BRCA2 modulates the early steps of repair mediated by the Rad50 nuclease complex, and the later stages catalyzed by the Rad51 recombinase. To test our hypothesis, we proposed to accomplish the following specific aims. Aim1. To establish the specificity of the interaction between Rad50, RINT-1 and BRCA2. Aim2. To determine how BRCA2 influences double strand break repair machinery. We found that human RINT-1 shares sequence homology with a novel protein identified in Drosophila melanogaster, including a coiled-coil domain within its N-terminal 150 amino acids, a conserved central domain of about 350 amino acids, and an C-terminal region of 90 amino acis exhibiting 35-38% identity. While Rad50 and RINT-1 are both expressed throughout the cell cycle, RINT-1 specifically binds to Rad50 only during late S and G2/M phases, suggesting that RINT-1 may be involved in cell cycle regulation. The characterization of RINT-1 protein represents an important step toward our goal in uncovering the significance of the interaction between BRCA2, RINT-1 and Rad50 nuclease complex.

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Introduction

The overall goal of my grant proposal is to understand the cellular function of BRCA2, and to elucidate its role in the development and progression of breast cancer. Recent studies have indicated that BRCA2 interacts with the Rad51 recombinase (Chen et al., 1998; Mizuta et al., 1997; Sharan et al., 1997) and that homozygous deletion of BRCA2 results in phenotypes highly similar to those observed when *RAD51* is deleted (Sharan et al., 1997). These data provide appealing clues that BRCA2 participates in the Rad51-dependent pathway of homologous recombination and double-strand break DNA repair. They further suggested those abnormalities in recombination and repair resulting from mutational inactivation or BRCA2 lead to the development of breast cancer. In my studies, I have uncovered strong evidence that BRCA2 has a dual role in double-strand break DNA repair. In my preliminary studies of the C-terminal region of BRCA2, I discovered that this domain is responsible for indirect interactions with another member of the DNA repair machinery, Rad50, through a novel protein that I isolated and named RINT-1 for Rad50 -interacting protein 1. Rad50 is a member of a larger complex containing Mre11 and p95, which together is postulated to possess endonuclease activity important in the early stages of end-processing of DNA breaks (Shinohara et al., 1995). In this model, BRCA2 modulates the early steps of repair mediated by the Rad50 nuclease complex, and the later stages catalyzed by the Rad51 recombinase. A central tenet of this novel idea is that BRCA2 has a positive effect on these early and late activities in repair, and that defects in their interaction is a contributing factor toward breast tumorigenesis and/or progression. To test our hypothesis, we will accomplish the following specific aims. Aim 1. To establish the specificity of the interactions between Rad50, RINT-1 (previously named RLB1) and BRCA2. Aim 2. To determine how BRCA2 influences double strand break repair machinery. Demonstrating a dual role for BRCA2 in the DNA-repair process represents a paradigm shift concerning its involvement in tumorigenesis and how this knowledge can be exploited toward the development of new strategies for its prevention and treatment of breast cancer.

Body

We have made the following progress on testing the novel idea that BRCA2 has a *dual* role in double-strand break repair.

Database searches revealed that hsRINT-1, has significant sequence homology with the CG8605 gene product in *Drosophila melanogaster*, which we refer to as dmRINT-1 (Fig.1). The predicted sequences of both of these proteins share similar leucine heptad repeats within their corresponding N-terminal region (box A). Two additional regions designated box B, which spans amino acids 220 to 565 of hsRINT-1 and box D, which spans amino acids 659 to 751 of hsRINT-1, exhibit 35-39% homology to the corresponding regions of dmRINT-1. hsRINT-1 has a unique sequence, box C, that lies between the two conserved regions. The biological function of the dmRINT-1 is unknown at the present time. A search of the NCBI human genome database revealed that hsRINT-1 maps to chromosome segment 7q22.1, between marker D7S2545 and D7S2420. Interestingly, deletion of this region is frequently associated with acute myeloid leukemia, myelodysplastic syndrome, breast and ovarian adenocarcinoma, and additional cancer.

1) To establish the specificity of the interactions between Rad50, RINT-1 and BRCA2 by:

a. **Yeast two-hybrid and *in vitro* binding assays to map the domains for interactions between Rad50, RLB1 and BRCA2.**

To test whether RINT-1 specifically binds to Rad50, we made three bait plasmids, pAS-R50PB, pAS-R50PB0.6 and pAS-R50PB1.2 that express the second leucine haptad repeat and the Walker B domain of Rad50, respectively, were constructed as yeast GAL4 DNA-binding domain fusions (Fig. 2A). These plasmids were individually co-transformed into *S. cerevisiae* Y153 with a prey plasmid pSE-RINT-1 that expresses full length RINT-1. Colorigenic and β-galactosidase activity assays (Durfee et al., 1993) indicated that the second leucine heptad repeat, but not the Walker B domain, of Rad50 interacts with RINT-1 (Fig. 2A). In vitro binding assays using GST fusions of the same regions of Rad50 gave similar results (Fig. 2C). To identify the domain of RINT-1 that interacts with Rad50, a numbers of RINT-1 deletion mutants were engineered as fusions with the GAL4 activation domain, and each mutant was tested for its ability to interact with Rad50, expressed from pAS-Rad50PB, following co-transformation in yeast. Resultant β-galactosidase assays revealed that the B, C and D region of the RINT-1 protein, corresponding to amino acids 256 to 792, is required for its interaction with Rad50 (Fig. 2B). Further deletions of either RINT-1 B or D region diminished interaction with Rad50 (Fig. 2B). We are in the process of making bait plasmids to map the regions in BRCA2 and Rint-1.

b. Testing the *in vivo* interactions between RINT-1, BRCA2 and the Mre11/Rad50/p95 complex using reciprocal co-immunoprecipitation

during cell cycle progression and subsequent to treatments of cells with DNA-damaging agents.

We have examined the expression profile of the RINT-1 protein doublet in a panel of human cell lines. These include a breast epithelial immortalized line MCF10A, three human breast cancer lines, T47D, MB231 and ZR75, two human bladder carcinoma lines, T24 and 5637, a rectal carcinoma line, SW837, and two immortalized fibroblast lines, GM09607A and GM09637G, carrying mutated and wild-type *ATM* gene, respectively. RINT-1 doublet was detected in all cell lines (Fig. 3), indicating that RINT-1 is expressed in many different kinds of human cells.

To further explore the interaction of RINT-1 and Rad50 in cells, human bladder carcinoma T24 cells synchronized by density arrest and subsequent release were harvested at different time points. Cell lysates were subjected to immunoprecipitation with anti-RINT-1 or anti-Rad50 antibody, followed by immunoblot analysis using antibodies specific for RINT-1 and Rad50. The specific cell cycle stage(s) from which the lysates were prepared at each time point was determined by probing immunoblots with anti-RB mAb as previously described (Chen et al., 1996). The amount of total lysate used for each time point was normalized by immunoblotting with an antibody specific for a nuclear matrix protein, p84 (Fig. 4, bottom panel)(Durfee et al., 1994). While the 87 kDa doublet was detected in immunoprecipitates using anti-RINT-1 antibody during all stages of the cell cycle, an interaction with Rad50 was observed only during late S, G2/M and M phases (lanes 4, 5, and 6 in the top panel). Reciprocally, an interaction was also detected only after late S phase by immunoprecipitation with anti-Rad50 mAb. Interestingly, both bands of the RINT-1 doublet were co-immunoprecipitated with Rad50 although the slower migrating form of RINT-1 may preferentially bind to Rad50. Nevertheless, the specific interaction between RINT-1 and Rad50 at late S and G2/M suggests that RINT-1 may play a role at these time windows during cell cycle progression.

The results suggested that RINT-1 is expressed throughout the cell cycle but interacts with Rad50 only at late S and G2/M phase. We will use similar approaches to determine the interaction between BRCA2 and RINT-1 during cell cycle progression.

c. Determining the spatial and temporal relationships of these interactions in cells.

To accomplish this task, we are in the process of making GFP fusing proteins and monoclonal antibodies specifically recognize RINT-1. These reagents will then be used to determine the spatial and temporal relationships of these interactions in cells.

d. Exploring the potential signal transduction pathway(s) leading to the formation of the RLB1/BRCA2/Mre11/Rad50/p95 complex.

Once we accomplish tasks **1a** and **1b**, the *in vivo* interaction between BRCA2, RINT-1 and Rad50 complexes will be determined. The results will provide parameters essential for task **1d**.

2) *To determine how BRCA2 influences double-strand break repair machinery by:*

a. Purifying recombinant RINT-1 from eukaryotic cell expression system.

We proposed to use both yeast and baculovirus systems to express his-tagged proteins and nickel chromatography for the purification of RINT-1. The full-length cDNAs encoding His-tagged RINT-1 has been subcloned into the baculovirus expression vector, pAcHLT, for expression and purification from insects cells. High titer baculoviral stock has been generated and will be used to infect Sf9 cells for the production of recombinant RINT-1 protein.

b. To test for RINT-1's effect in modulating the endonuclease activities of the Mre11/Rad50/p95 complex.

We proposed to examine the kinetics of the nucleolytic products generated by the Mre11/Rad50/p95 complex both in the presence and absence of RINT-1. To facilitate the production of Mre11/Rad50/p95 complex, we have also generated baculoviral stocks for all three proteins. Recombinant Mre11/Rad50/p95 will be purified from insect cell lysates using standard chromatographic protocols and then by nickel chromatography.

c. BRCA2 protein will be expressed and purified from baculovirus or yeast expression systems.

We tried to express a His-tagged BRCA2 recombinant protein in insect cells, and purify it using biochemical fractionation and nickel chromatography. The 370KD recombinant BRCA2 protein suffered from proteolytic degradation during purification process. Furthermore, the N-terminal His-tagged BRCA2 didn't bind the nickel column efficiently. To circumvent these problems, we are in the process of making c-terminal HA-tagged BRCA2 cDNA. The engineered BRCA2 cDNAs will then be subcloned into the baculovirus expression vector, pAcHLT, for expression and purification from insect cells.

d. To test of the ability of BRCA2 to influence a defined biochemical activity.

Once the difficulties we encountered in task **2c** are overcomed, we shall proceed the experiments as proposed.

Key Research Accomplishments

1. RINT-1encodes a novel human protein that shares a significant homology with a putative Drosophila protein
2. RINT-1 interacts specifically with Rad50 in yeast two hybrid and GST-pull down assays.
3. RINT-1 is expressed throughout the cell cycle but interacts with Rad50 only at late S and G2/M phase.

Reportable outcomes

The research conducted in this grant proposal has resulting a paper accepted for publication in JBC. The paper entitled "RINT-1, a novel Rad50-interacting protein, participates in radiation-induced G2/M checkpoint control".

Conclusions

Since the beginning of grant funding, we have characterized RINT-1 in great detail. We found that human RINT-1 shares sequence homology with a novel protein identified in *Drosophila melanogaster*, including a coiled-coil domain within its N-terminal 150 amino acids, a conserved central domain of about 350 amino acids, and an C-terminal region of 90 amino acids exhibiting 35-38% identity. The conserved central and C-terminal regions of RINT-1 are required for its interaction with Rad50. While Rad50 and RINT-1 are both expressed throughout the cell cycle, RINT-1 specifically binds to Rad50 only during late S and G2/M phases, suggesting that RINT-1 may be involved in cell cycle regulation. We are in the process of examining the interaction of BRCA2 with RINT-1 protein using reciprocal co-immunoprecipitation during cell cycle progression and subsequent to treatments of cells with DNA-damaging agents. These are straightforward experiments; we don't anticipate any difficulties in these experiments. Moreover, various high titer baculoviral stocks, including those of RINT-1, Rad50, Mre11 and NBS1, were generated. We are now ready to proceed to purify each protein required in experiments proposed in Aim2.

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Appendices

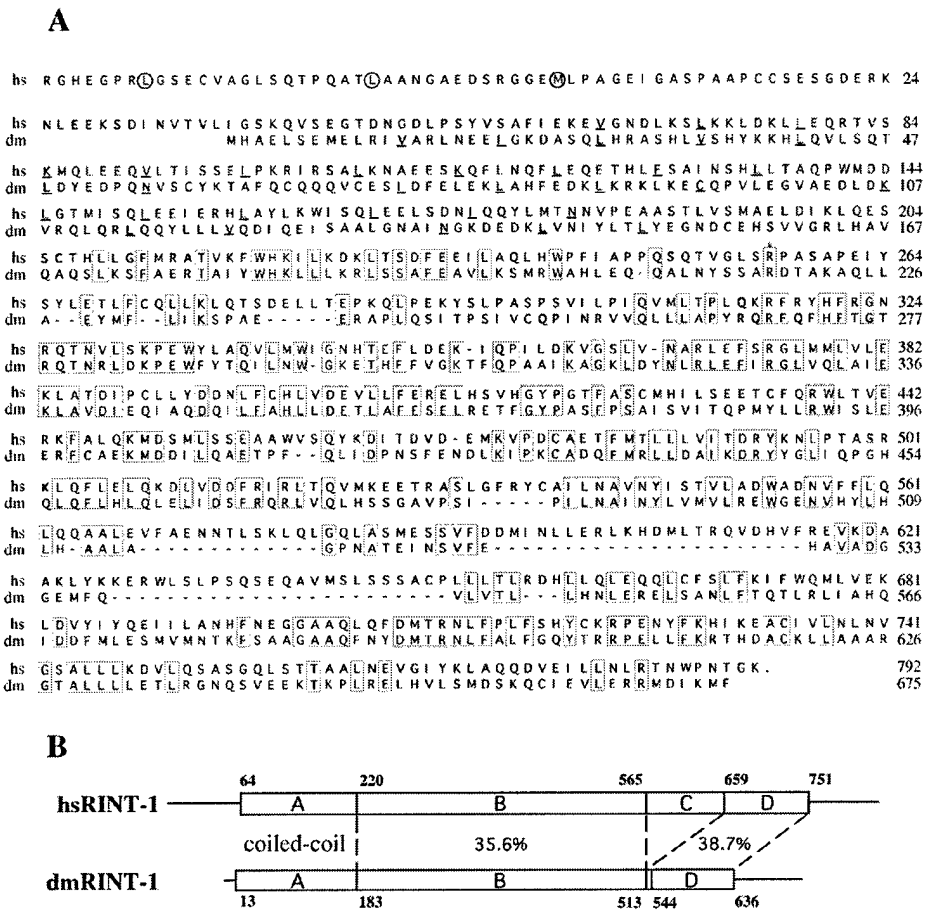

FIG. 1. **Sequence comparison between hsRINT-1 and dmRINT-1.** *A*, alignment of the predicted amino acids sequence of hsRINT-1 and dmRINT-1. dmRINT-1 is a deduced protein sequence from the CG8605 gene product of *Drosophila melanogaster*. Circled residues indicate the potential initiation codons that include first methionine site and two potential non-AUG translational start sites at leucines. The residues demarcating the leucine heptad repeats are underlined. Boxed residues represent identical residues between the two species. The RINT-XS-GFP fusion protein in Fig 5 includes RINT-1 protein sequences initiating at arginine residue 256 marked with star. *B*, schematic diagrams showing homologous regions between human and fly RINT-1. Box A represents the region containing leucine heptad repeats of hsRINT-1 (amino acids 64 to 220) and dmRINT-1 (amino acids 13 to 183). Box C represents the unique region of hsRINT-1 (amino acids 565 to 659). Protein sequence similarities between box B and box D of human and fruit fly are 35.6% and 38.7%, respectively.

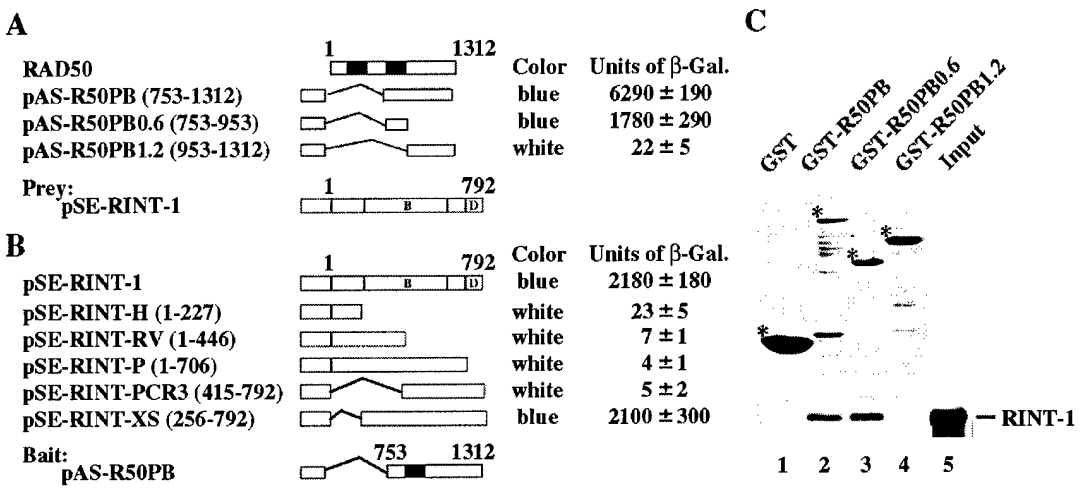

FIG. 2. **Interaction between RINT-1 and Rad50.** *A*, hRad50 binds RINT-1 in a yeast two-hybrid assay. The indicated regions of hRad50 were fused to the DNA-binding domain of GAL4 in pAS1. RINT-1 was fused to the activation domain of GAL4 in pSE1107. These plasmids were co-transformed into yeast strain Y153, β-galactosidase activity was assayed and quantified with the chlorophenol red-β-D-galactopyranoside (CPRG). Both of the leucine heptad repeats of hRad50 are represented as solid rectangles. The result indicates that the second heptad repeat of Rad50 specifically binds to RINT-1. *B*, RINT-1 binds hRad50. The indicated regions of RINT-1 were fused to the activation domain of GAL4 in pSE1107, and these plasmids were cotransformed with pAS-R50PB containing amino acids 753-1312 of hRad50 as in (*A*). Note that deletions of amino acids in the conserved region of RINT-1 (box B and D) abrogated its binding to hRad50 in this assay. *C*, hRad50 binds RINT-1 by GST pull-down assay. The expressed and purified hRad50-GST fusions are shown in the upper panel. Stars indicate each corresponding full-length GST fusion protein. In vitro translated RINT-1 was used to bind to GST-fusions and the results are shown in the lower panel. The R50PB (amino acids 753-1312) and R50PB0.6 (amino acids 753-953) derivatives bind to RINT-1 (lanes 2 and 3) but not the GST or R50PB1.2 (lanes 1 and 4) proteins. Lane 5 shows ten percent of the total input of the in vitro translated RINT-1.

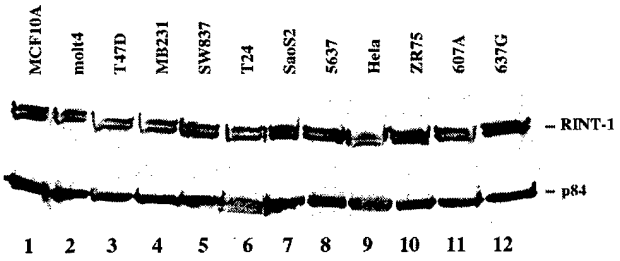

FIG. 3. **The RINT-1 protein is expressed in various cell-types.** Lysates prepared from the indicated cell lines were directly immunoblotted with anti-PIN antibodies. p84, which is a nuclear matrix protein, was used to normalize total protein amount.

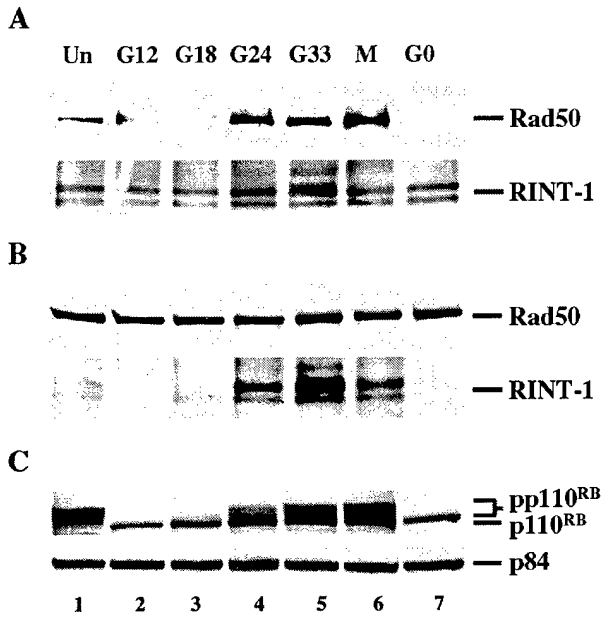

FIG.4. **Cell cycle-dependent interaction between RINT-1 and Rad50.** Unsynchronized human bladder carcinoma T24 cells (Un) were arrested at G_0 by contact inhibition (G0), and released by plating at low density for various time periods (G12 to G33, indicating hours after release from G_0 arrest). M phase cells were prepared after release for 24 hours followed by treatment with nocodazole. Lysates of T24 cells at each time point were immunoprecipitated with anti-RINT-1 (*A*) or anti-Rad50 (*B*) antibodies. The immunoprecipitates were immunoblotted with anti-RINT-1 or anti-Rad50 antibodies, respectively, (*A* and *B*). *C*, the lysates were also immunoblotted with anti-Rb mAb to verify the cell cycle status at each time point, and with anti-p84 mAb as a loading control. The RINT-1 doublets were coimmunoprecipitated with Rad50 during late S (G24), G2/M (G33), and M phases.